

Anthocyanin-rich Extracts Affect Coaggregation Among Oral Bacteria

Monica Bonnin

INTRODUCTION

Bacteria colonize the mouth within minutes after birth and form stable, organized communities on teeth, mucosa and under the gums¹. These bacterial communities are called commensal biofilms and are important in maintaining oral health². Initiation of bacterial colonization begins with bacterial interactions in which genetically distinct bacteria adhere together in a process known as coaggregation^{3,4}. Periodontal disease is initiated when the commensal biofilms are dominated by pathogens; these pathogenic bacterial consortia lead to destruction of structures that support tooth attachment to the jawbone^{1,5}. One half of the American population ≥ 30 years of age is estimated to have periodontitis⁶.

Dietary sources are becoming a popular area of interest due to all the natural occurring compounds with potential health benefits⁷. Without the necessity of synthesizing, extracting the health promoting molecules from natural plants, fruits, or vegetables are of interest.

Anthocyanins (ACN) are water-soluble pigments present in various fruits such as black raspberries, strawberries, and red grape. ACN have been suggested to have health promoting activities such anti-inflammatory, anti-obesity, and anti-cancer effects⁸⁻¹⁰. Previous studies have proposed that ACN may exert a significant anti-bacterial effect¹¹. Several studies suggest that antimicrobial activity from anthocyanins is capable of inhibiting gram negative rather than gram positive bacteria¹². Four anthocyanins Pg, Cy, Dp, and Cy-3-glu were discovered to be inhibitors of gram-negative *Escherichia coli* strain CM 871, a DNA repair-deficient strain, but did not inhibit regular *E. coli* and the beneficial Gram-positive probiotic bacteria¹³. Thus the antimicrobial activity is speculated to involve reactions related to DNA and that anthocyanins' effects are species specific. Additionally the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that anthocyanin-containing extracts have a very low toxicity¹⁴. Therefore the therapeutic usage of anthocyanins is advocated to be safe, and with the easy accessibility, ACNs may be considered for prevention or for treatment of oral diseases.

Anthocyanins are polyphenol compounds that originally occur as glycosides of their aglycone anthocyanidin-chromophores with the sugar usually attached at the 5,7-position on the A-ring or 3-position on the C-ring. The most distributed anthocyanidins are cyaniding (Cy), peonidin (Pn), pelargonidin (Pg), malvidin (Mv), delphinidin (Dp), and petunidin (Pt). The most common observed sugars attached to the anthocyanidin structure as mono-, di-, or tri- saccharide forms are the following: glucose (glc), galactose (gal), arabinose (arab), rutinose (rut), rhamnose (rham), and xylose (xyl). There are over 600 different anthocyanins due to their variation in the number and position of hydroxyl and methoxyl groups on the ACN structure and also the identity, number, and position of the sugar.

In our current investigation ACN from red grape, strawberry, and black raspberry are being studied to observe its effects on oral bacteria co-aggregation, which is the first initial step of biofilm formation. We are characterizing the effects of ACN-rich extracts from these fruits on both the viability and co-aggregation of common oral bacterial species. Red grape, strawberry, and black raspberry have been selected for this study because of their varying structures of anthocyanins they contain. Red grape includes five different anthocyanins which are Dp-3-glu, Cy-3-glu, Pt-3-glu, Pn-3-glu, and Mv-3-glu. Strawberry has three anthocyanins Cy-3-glu, Pg-3-glu, and Pg-3-rut. Black raspberry only has two anthocyanin present which are Cy-3-(2G-xyl-rut) and Cy-3-rut. Note that Cy-3-glu is present in both strawberry and red grape.

We hypothesized that ACN will then have the capability to effect co-aggregation to early stages of biofilm formation. The purpose of this study was to characterize the effects of anthocyanin-rich extracts on auto-and co-aggregation on oral bacteria and early-stages of biofilm formation. ACN-rich extracts derived from natural food products have potential to modulate formation of biofilms. The strategic use of ACN-rich exacts for promotion of oral health as a co-adjuvant for the treatment of periodontal disease, a risk factor for oral cancer is being considered. This study applies a quantitative spectrophotometric assay with multiphoton confocal microscopy to investigate oral bacteria co-aggreaction interactions and in early stages of biofilm formation. To the best of our knowledge, this is the first time oral bacteria have been treated with ACN in the processes of co-aggregation and biofilm formation.

METHODS

Auto- and Co-aggregation

Commensal species *Veillonella parvula*, *Actinomyces naeslundii*, *Streptococcus oralis*, *Streptococcus sanguis*, *Streptococcus mitis*, and bridging species *Fusobacterium nucleatum* were grown in Brain Heart Infusion (BHI) for 48 hours. The bacteria were then washed and resuspended with Phosphate Buffered Saline (PBS) and measured to an optical density (OD) of 1.0. For non-anthocyanin treated assays, equal volumes of bacteria (150µL) were paired into 96-well plates. For the anthocyanin treated bacteria, equal volumes of bacteria (125µL) were paired into 96-well plates and 50µL of black raspberry, red grape, or strawberry extracts were added¹⁵. After the initial OD was taken, the bacterial pairs were transferred to test tubes and shaken at room temperature for 1 hour. Bacteria pairs were then transferred back to a 96-well plate to measure the OD. These same steps were then repeated with pathogen species *Capnocytophaga ochracea*, *Porphyromonas gingivalis*, *Parvimonas micra*, *Tannerella forsythia* and bridging species *Fusobacterium nucleatum*. The assays were ran in triplicate.

Biofilm growth

Commensal species *Veillonella parvula*, *Actinomyces naeslundii*, *Streptococcus oralis*, *Streptococcus sanguis*, *Streptococcus mitis*, and bridging species *Fusobacterium nucleatum* were grown in Brain Heart Infusion (BHI) for 48 hours. Bacteria were standardized to an optical density (OD) of 1.2. In a biofilm set up (glass slide inside a 50 mL test tube), commensal bacteria were suspended in 1:1 BHI and artificial saliva for a total of 30 mL. For biofilms treated with ACN, an additional 100 µL of strawberry, red grape, or black raspberry ACN were added to the biofilms. Biofilms were grown in an aerobic incubator and after 24 hrs, fed 10 mL of BHI as well as an additional 100 µL of ACN to the ACN-treated biofilms. After 48 hours, media was removed and fresh 1:1 BHI and artificial saliva was added with secondary and tertiary colonizers (*Capnocytophaga ochracea*, *Porphyromonas gingivalis*, *Parvimonas micra*, *Tannerella forsythia* and bridging species *Fusobacterium nucleatum*) standardized to an OD of 1.2. ACN-treated biofilms had 100 µL of ACN added to maintain the treatment. After 24 hrs, biofilms were fed 10 mL of BHI and ACN-treated biofilms had 100 µL of ACN added. Biofilms were imaged after another 24 hrs of growth. The assays were run in triplicate.

Imaging

After the first 48 hrs the biofilms with primary colonizers were imaged. The glass slide was removed and washed in a 15 mL NaCl filled petri dish. The glass slide was then placed in another petri dish with 5 mL of NaCl where it was stained with the Live/Dead BacLight Bacterial Viability Kit. The glass slides were then imaged with an Olympus FV1000 Multiphoton. After the second 48 hrs the biofilms with secondary and tertiary colonizers were imaged using the same procedure detailed above.

Analyses

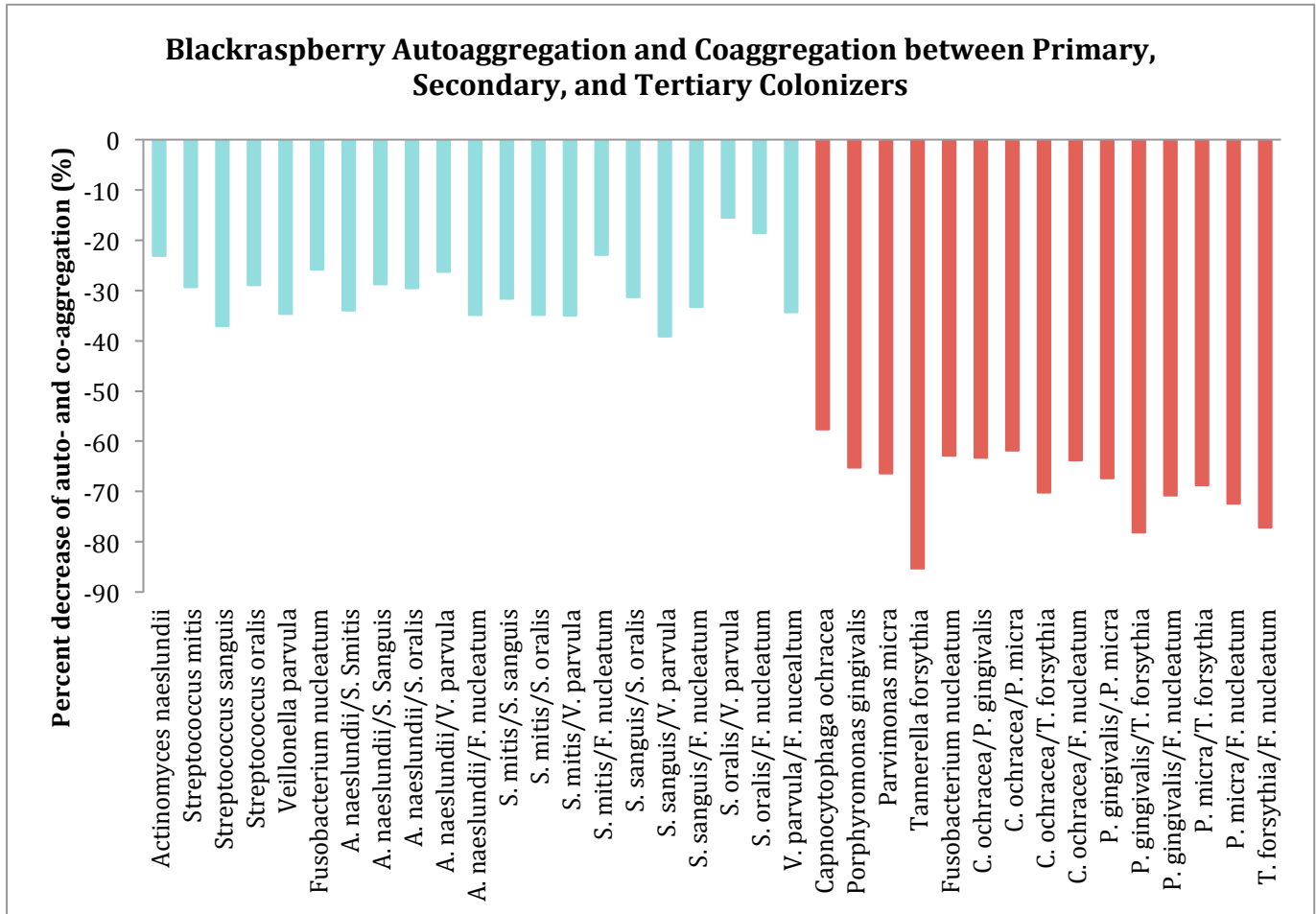
The amount of co-aggregation was expressed as a function of the control samples. Pairwise comparisons were made between anthocyanin-conditioned and anthocyanin free coaggregation.

RESULTS

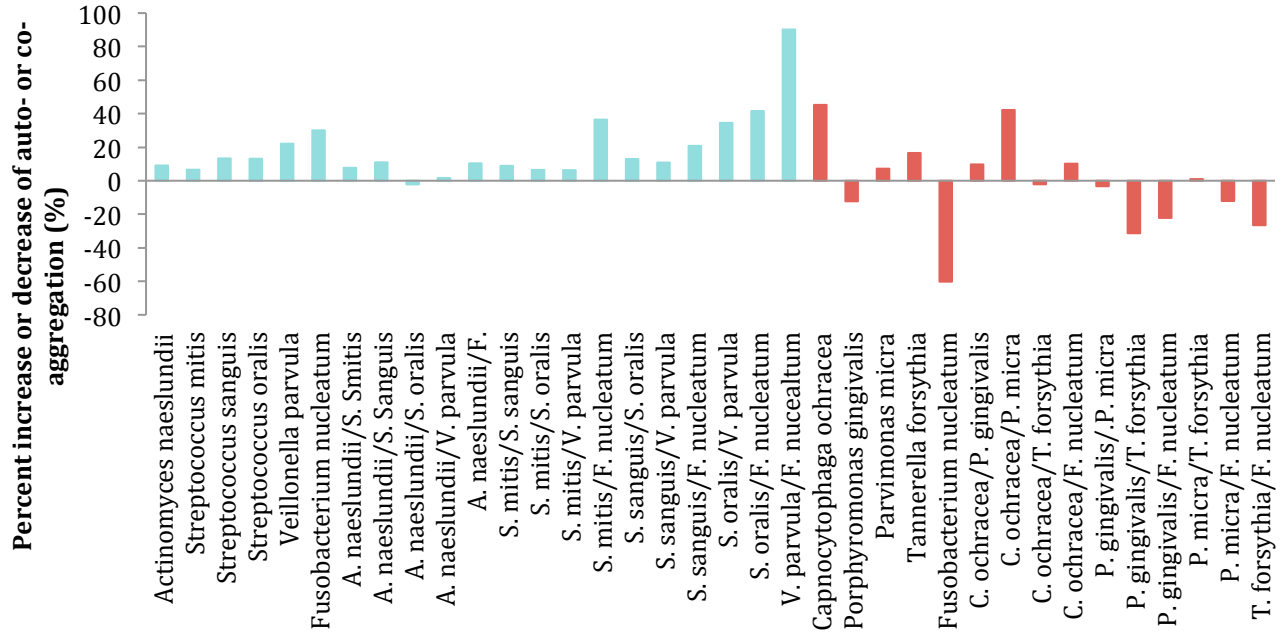
Auto- and co-aggregation

The addition of ACN among oral commensal and pathogenic bacteria led to differing results between the ACN derived from strawberries, black raspberries, and red grape.

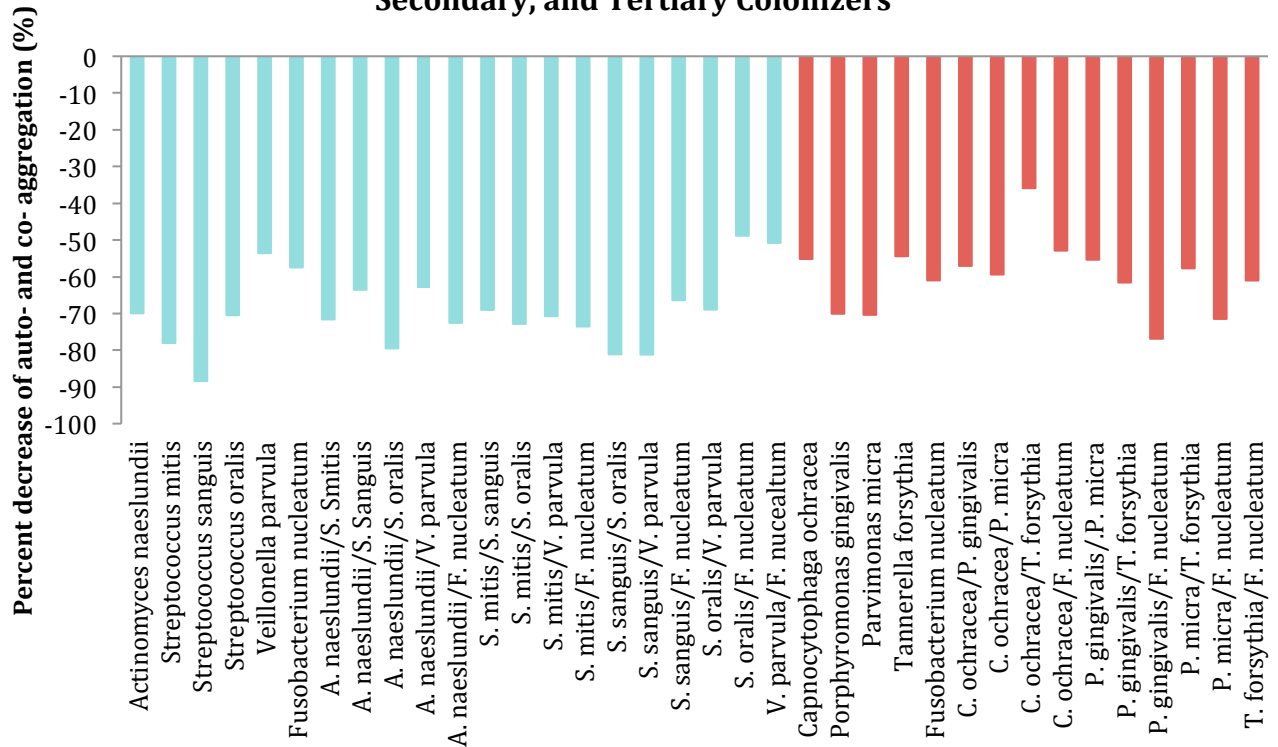
Below are the auto- and co-aggregation assays for the different fruit derived ACNs. The blue bars refer to commensal bacteria and red bars depict the pathogens.



Strawberry Autoaggregation and Coaggregation between Primary, Secondary, and Tertiary Colonizers



Redgrape Autoaggregation and Coaggregation between Primary, Secondary, and Tertiary Colonizers



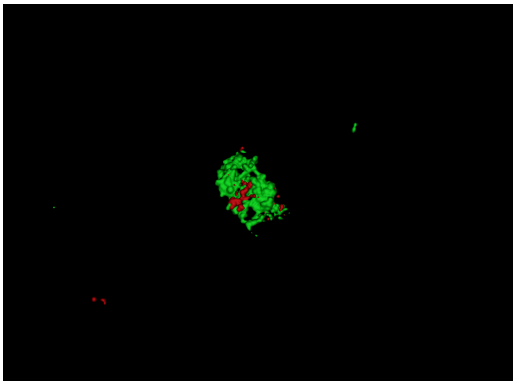
From the graphs, Black Raspberry demonstrated to have a greater extent of inhibiting pathogens than commensals when comparing to controls. Black raspberry showed to inhibit commensals coaggregation by 30.0% and inhibited pathogenic coaggregation by 68.7%. Black raspberry proved to be specie specific where its affects differ from commensals and pathogens.

Strawberry was distinct from the other ACN because it had the ability to increase and decrease co-aggregation depending on the species. Strawberry increased co-aggregation between all commensals except the interaction between *Actinomyces naeslundii* and *Streptococcus oralis* where there was a 2.2% decrease of coaggregation. Strawberry demonstrated to be effective to auto-aggregation of *Capnocytophaga ochracea* (45.2% increase) and *Fusobacterium nucleatum* (60.2% decrease).

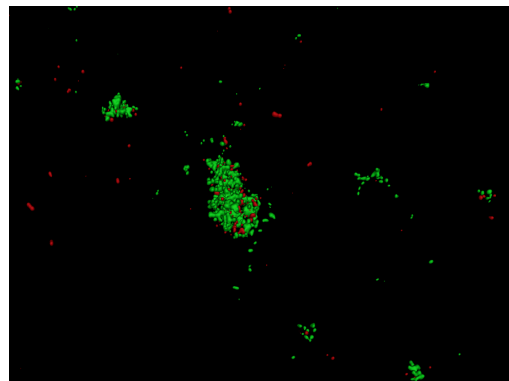
Redgrape exhibited the potential to decrease auto-and co-aggregation of both commensals and pathogens. Red grape inhibited all bacterial pairings by at least 35.9% decrease. Commensal bacteria co-aggregation were found to decrease with an average of 69.1% and pathogen bacteria co-aggregation were found to decrease with an average of 60.0%.

Biofilm imaging

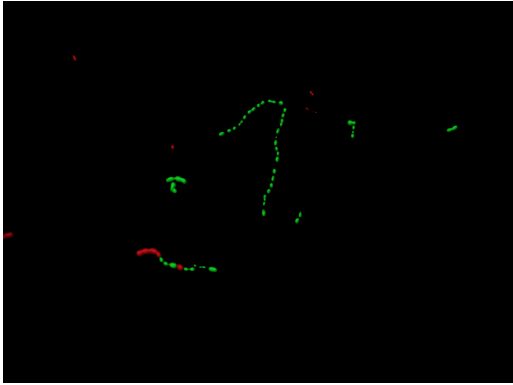
Below are the images of the first 48 hours of the early stages of biofilm formation with commensals.



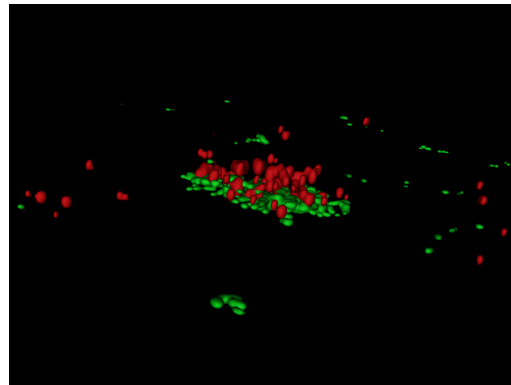
Control



Black Raspberry

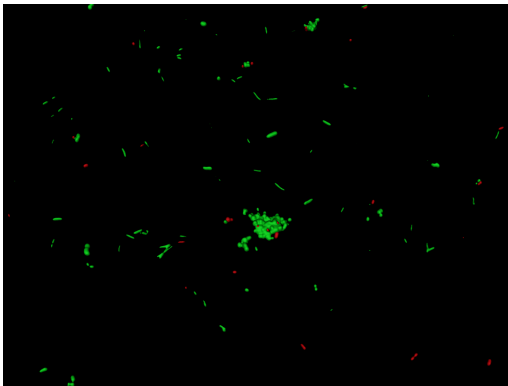


Red Grape

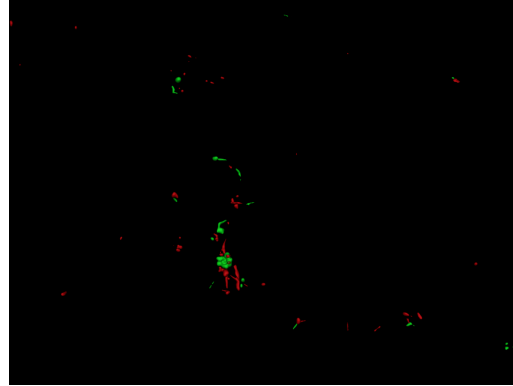


Strawberry

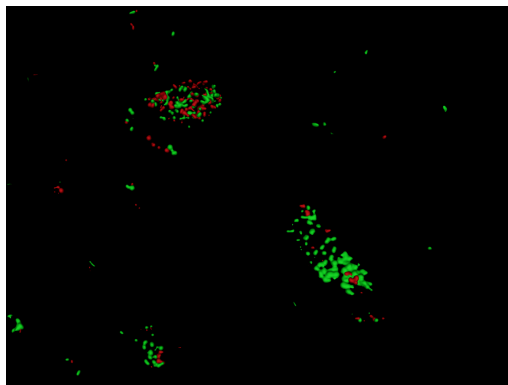
Below are the images 48 hours after the addition of pathogens added to the biofilms.



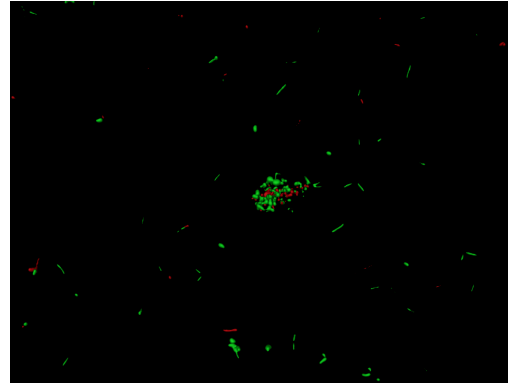
Control



Black Raspberry



Red Grape



Strawberry

The images show ACN do not affect bacteria viability, but do modulate the biofilm structures.

DISCUSSION

Due to galactosides behaving as competitive inhibitors of coaggregation, a lectin-carbohydrate recognition between the associated bacteria on the two cell surfaces is suggested. Thus when bacteria are treated with ACNs, the varying sugars attached to the ACNs may mediate the coaggregation involvement.

Because strawberry's Pg-3-glu and Pg-3-rut had the ability to increase auto- and co-aggregation with certain species, these ACN with their respectable sugar may have been recognizable cognate molecules to the lectin-receptor which led them to cumulative adherences. Black Raspberry contains Cy-3-(2G-xyl-rut) and Cy-3-rut therefore both strawberry and black raspberry share rutinose as a common sugar. However the combination of rutinose with the cyanidin anthocyanidin backbone in black raspberry decreased coaggregation. Red grape with its five ACN had the ability to decrease coaggregation which may be due to its abundant sugar glucoside in all its ACN. Red grapes reoccurring glucoside may be an undistinguishable sugar to the lectin-receptor. It should be noted that strawberry's third ACN present is Cy-3-glu which is also present in red grape. Therefore strawberry's incapacity of increasing coaggregation with *Fusobacterium nucleatum* and between *Porphyromonas gingivalis* and *Tannerella forsythia* may be due to Cy-3-glu. Consequently the lectin-receptor is specific where the sugar identity and attachment on particular compounds is noteworthy.

Hydroxyl groups have also been shown to facilitate coaggregation on the bacteria's ability to identify certain cell surfaces. Studies have suggested that sugars such as L-rhamnose and N-acetyl-D-glucosamine have demonstrated no inhibitory affects. This may be due to their hydroxyls on C-3 and C-4 which are in the *trans* configuration. Strawberry's Pg-3-rut displays several hydroxyls on the sugar where the neighboring hydroxyls are also in *trans* configuration which may explain strawberry's ability to increase coaggregation. Black Raspberry's Cy-3-rut also displays *trans* configuration of hydroxyls because it also contains the same sugar. However black raspberry decreased coaggregation, leading to the conclusion that hydroxyl configuration does play a factor in cell bonding—in alignment with the backbone of the ACN. Red grape contains glucoside which also displays *trans* configuration yet decreased coaggregation. Interestingly three of the ACN present in red grape contain methoxy groups attached to its backbone (Pt-3-glu, Pn-3-glu, and Mv-3-glu), which is not seen in either strawberry or black raspberry. Methoxys on the backbone structure of ACN is then under consideration with its abilities of inhibition. Thus *trans* configuration with neighboring hydroxyls and certain ACN backbone structures may be a recognition site aiding in the primary steps of coaggregation. The ACN backbone however may have a greater effect towards the cell receptors.

The differences between different fruits thus distinctive ACN composition, may have alternating effects on coaggregation due to their structural differences of the sugar, moiety of hydroxyl groups, and structure of the backbone. Methoxys, hydroxyls, and sugars are suggested to play a role in the interaction between bacteria's binding receptors including the lectin-dependent receptor.

Further investigation is necessary to understand the full potential of ACN in the oral cavity. DNA sequencing is the next step to better characterize the effects of ACNs on oral bacteria, and to observe which bacterial species are able to participate in the biofilm assembly under ACN treatments. Afterwards testing *in vivo* would be applicable in order to examine the efficacy of ACN in the human oral cavity.

REFERENCES CITED

- 1 Socransky, S. S. & Haffajee, A. D. Periodontal microbial ecology. *Periodontol 2000* **38**, 135-187 (2005).
- 2 Darveau, R. P., Tanner, A. & Page, R. C. The microbial challenge in periodontitis. *Periodontol 2000* **14**, 12-32 (1997).
- 3 Kolenbrander, P. E. *et al.* Bacterial interactions and successions during plaque development. *Periodontol 2000* **42**, 47-79 (2006).
- 4 Kolenbrander, P. E. & Palmer, R. J. J. in *Microbial Biofilms* (eds M. Ghannoum & G. O'Toole) 85-117 (ASM Press, 2004).
- 5 Socransky, S. S. *et al.* The microbiota of the gingival crevice area of man. I. Total microscopic and viable counts and counts of specific organisms. *Arch Oral Biol* **8**, 275-280 (1963).
- 6 Eke, P. I. *et al.* Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J Dent Res* **91**, 914-920, doi:10.1177/0022034512457373 (2012).
- 7 Scalbert, A. & Williamson, G. Dietary intake and bioavailability of polyphenols. *J Nutr* **130**, 2073S-2085S (2000).
- 8 Mallery, S. R. *et al.* Effects of human oral mucosal tissue, saliva, and oral microflora on intraoral metabolism and bioactivation of black raspberry anthocyanins. *Cancer Prev Res (Phila)* **4**, 1209-1221, doi:1940-6207.CAPR-11-0040 [pii]
10.1158/1940-6207.CAPR-11-0040 (2011).
- 9 Ugalde, C. M. *et al.* Distribution of anthocyanins delivered from a bioadhesive black raspberry gel following topical intraoral application in normal healthy volunteers. *Pharm Res* **26**, 977-986, doi:10.1007/s11095-008-9806-x (2009).
- 10 Zikri, N. N. *et al.* Black raspberry components inhibit proliferation, induce apoptosis, and modulate gene expression in rat esophageal epithelial cells. *Nutr Cancer* **61**, 816-826, doi:10.1080/01635580903285148 (2009).
- 11 Sanchez-Patan, F. *et al.* In Vitro Fermentation of a Red Wine Extract by Human Gut Microbiota: Changes in Microbial Groups and Formation of Phenolic Metabolites. *Journal of agricultural and food chemistry*, doi:10.1021/jf2040115 (2012).
- 12 Cisowska, A., Wojnicz, D. & Hendrich, A. B. Anthocyanins as antimicrobial agents of natural plant origin. *Natural product communications* **6**, 149-156 (2011).
- 13 He, J. *Isolation of anthocyanin mixtures from fruits and vegetables and evaluation of their stability, availability and biotransformation in the gastrointestinal tract*, Ohio State University, (2008).
- 14 Clifford, M. N. Anthocyanins – nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture* **80**, 1063-1072, doi:10.1002/(SICI)1097-0010(20000515)80:7<1063::AID-JSFA605>3.0.CO;2-Q (2000).
- 15 Kamonpatana, K. *et al.* Susceptibility of anthocyanins to ex vivo degradation in human saliva. *Food Chem* **135**, 738-747, doi:10.1016/j.foodchem.2012.04.110 (2012).